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ORIGINAL ARTICLE

Inactivation of tumor-specific CD8$^+$ CTLs by tumor-infiltrating tolerogenic dendritic cells

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Cancer immunosurveillance failure is largely attributed to the insufficient activation of tumor-specific class I major histocompatibility complex (MHC) molecule (MHC-I)-restricted CD8$^+$ cytotoxic T lymphocytes (CTLs). DEC-205$^+$ dendritic cells (DCs), having the ability to cross-present, can present captured tumor antigens on MHC-I alongside costimulatory molecules, inducing the priming and activation of tumor-specific CD8$^+$ CTLs. It has been suggested that reduced levels of costimulatory molecules on DCs may be a cause of impaired CTL induction and that some tumors may induce the downregulation of costimulatory molecules on tolerogenic DCs. To examine such possibilities, we established two distinct types of murine hepatoma cell lines, named Hepa1-6-1 and Hepa1-6-2 (derived from Hepa1-6 cells), and confirmed that they display similar antigenicities, as well as identical surface expression of MHC-I. We found that Hepa1-6-1 had the ability to grow continuously after subcutaneous implantation into syngeneic C57BL/6 mice and did not prime CD8$^+$ CTLs. In contrast, Hepa1-6-2 cells, which display reduced levels of adhesion molecules, such as Intercellular Adhesion Molecule 1 (ICAM-1), failed to grow *in vivo* and efficiently primed CTLs. Moreover, Hepa1-6-1-derived factors, such as transforming growth factor (TGF)-β1, vascular endothelial growth factor (VEGF) and α-fetoprotein (AFP), converted CD11c^{high} MHC-II^{high} DEC-205$^+$ DC subsets into tolerogenic cells, displaying downregulated costimulatory molecules and having impaired cross-presenting capacities. These immunosuppressive tolerogenic DCs appeared to inhibit the induction of tumor-specific CD8$^+$ CTLs and suppress their cytotoxic functions within the tumor. Together, the findings presented here provide a new method of cancer immunotherapy using the selective suppression, depletion or alteration of immunosuppressive tolerogenic DCs within tumors.
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The major effector cells for tumor elimination are CD8$^+$ cytotoxic T lymphocytes (CTLs) that specifically recognize tumor-derived antigenic epitopes in association with the major histocompatibility complex (MHC)-I of the syngeneic haplotype.[1] Such tumor-specific CD8$^+$ CTLs are known to be induced principally by autologous dendritic cells (DCs) that present antigenic epitopes from captured tumor-derived peptides on MHC-I alongside appropriate costimulatory molecules, such as CD80, CD86 and CD40.[2,3] However, it has been argued that epitope peptides will only be presented by MHC-I when the antigenic molecules containing the epitope are endogenously synthesized within the antigen-presenting cell (APC); in contrast, epitopes from exogenously captured antigenic molecules are usually presented by class II MHC (MHC-II), which can prime CD4$^+$ T cells when the MHC-II molecules associate with the appropriate costimulatory molecules on DCs.[4–6]

Recently, it has been demonstrated that two distinct subsets of DCs regulate immune responses *in vivo*: 33D1 DC inhibitory receptor-2$^+$ DCs[7] and DEC-205 (CD205$^+$ DCs).[8] The CTL epitopes from externally captured antigenic proteins seem to be presented by MHC-I via cross-presentation, particularly in DEC-205+ DCs.[9] Therefore, if tumor fragments are captured by neighboring DCs that have cross-presenting capacity, tumor-specific CD8$^+$ CTLs can be primed. It should be noted that cross-presentation can also be induced by immunization with antigenic molecules together with various adjuvants[10] or by stimulation of DC with captured-antigen-associated TLR3 ligand such as poly(I:C).[11,12] Indeed, we have previously demonstrated that tumor-specific CD8$^+$ CTLs can be induced by OVA-expressing EL4 thymoma (E.G7) cells through oral immunization with OVA and cholera toxin (CT).[13] Furthermore, it has recently been reported that a vaccine containing the known adjuvant immunostimulating complex elicited a high frequency of antigen-specific CD8$^+$ CTLs capable of tumor cell killing in different tumor models.[14,15]

In contrast, it has been demonstrated that herpes simplex virus infection[16] or treatment with mitomycin C[17] decreased the expression of costimulatory molecules (CD80 and CD86) on DCs, converting these cells into tolerogenic cells with the potential to suppress T-cell priming. It is possible that such tolerogenic DCs expressing reduced

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levels of costimulatory molecules could inhibit the stimulation of antigen-specific CD8$^+$ CTLs within certain tumors if the DCs were present in the tumor mass.[18] Indeed, it has recently been demonstrated that DCs from mice in the early stages of tumorigenesis were immunocompetent, having the ability to expand tumor-reactive T cells and suppress tumor growth. However, in the advanced stages of tumor progression, immunocompetent DCs were depleted from the mice and converted to tolerogenic cells, resulting in suppression of tumor-reactive T-cell expansion.[19] It should be noted that the epitope specificity for tumor-reactive T cells in both early and advanced tumor cells remained unchanged, suggesting that the DCs were converted during tumor progression without changing their antigenicity.

On the basis of these findings, we established two distinct hepatoma cell lines, Hepa1-6-1 and Hepa1-6-2 from Hepa1-6 cells.[20] Consistent with observations on advanced-stage tumors, Hepa1-6-1 cells grew rapidly and formed a solid tumor mass after subcutaneous (s.c.) implantation, whereas the growth of Hepa1-6-2 cells was suppressed for at least 7 days after s.c. implantation in the abdominal region of syngeneic C57BL/6 mice. Both Hepa1-6-1 and Hepa1-6-2 cell lines express the same antigenic epitopes and are presented by the same MHC-I. Furthermore, both tumor lines are targeted and depleted in equal numbers by a Hepa1-6-specific CD8$^+$ CTL line.[20,21] Moreover, although the suppression of Hepa1-6-2 growth *in vivo* was mediated by tumor-specific CD8$^+$ CTLs within tumor-infiltrating lymphocytes (TILs), such CD8$^+$ CTLs were not observed within the TILs of implanted Hepa1-6-1 tumors.

Using these two distinct hepatoma cell lines, we compared tumor-infiltrating DCs (TIDCs) in both tumors and confirmed that CD11c^{high} MHC-II^{high} DEC-205+ DC subsets were observed in TIDCs in both tumors. Nevertheless, although TIDCs within the Hepa1-6-2 compartment could efficiently prime specific CD8$^+$ CTLs, TIDCs within the Hepa1-6-1 tumor mass were tolerogenic, expressing reduced levels of costimulatory molecules and having impaired cross-presenting capacities, each of which could suppress the induction of CD8$^+$ tumor-specific CTLs, and thus tumor cell expansion, *in vivo*. Given these findings, we would like to propose a new method for immunotherapy using the selective suppression, depletion[22] or even alteration[23] of impaired tolerogenic CD11c^{high} MHC-II^{high} DEC-205+ TIDCs using adjuvants or TLR ligands.[24]

RESULTS

Establishment and characterization of two distinct cell lines, Hepa1-6-1 and Hepa1-6-2, from the previously characterized murine hepatoma cell line Hepa1-6

As described in the METHODS section, we established two distinct hepatoma cell lines, Hepa1-6-1 and Hepa1-6-2, from Hepa1-6 cells. Next, we examined the expression of the class I MHC molecules (MHC-I) Kb and Db and found that the expression of these molecules was similar on the Hepa1-6-derived tumor cell lines (Figure 1a). We then confirmed that both established hepatoma cell lines showed similar sensitivities to depletion by a previously described Hepa1-6-specific CD8$^+$ CTL line *in vitro*[20,21] (Figure 1b). Thus, these tumor cell lines share antigenic epitopes critical for recognition by the CTL line.

Unexpectedly, the growth of Hepa1-6-2, determined by ^{3}H-thymidine uptake, was faster than that of Hepa1-6-1 and Hepa1-6, and the expression of the cell adhesion molecule Intercellular Adhesion Molecule 1 (ICAM-1) was markedly downregulated in Hepa1-6-2 compared with Hepa1-6-1, which had similar ICAM-1 expression to the parent Hepa1-6 cell line (Figure 1c). Thus, the physiological characteristics of Hepa1-6 and Hepa1-6-1 are similar, at least when the cells are maintained in the D-10 medium. Additionally, it should be noted that the Hepa1-6-2 cells did not convert into Hepa1-6-1-like cells, even after being cultured in D-10 for several months. When the established hepatoma cell lines Hepa1-6-1 and Hepa1-6-2 were injected s.c. into the right abdominal region of syngeneic C57BL/6 mice, both tumors grew and established solid tumors; however, Hepa1-6-2 cells began to diminish ~7 days post implantation and were undetectable by 3 weeks (Figures 1d and e). These results indicate that the rapid growth of the Hepa1-6-2-dependent tumor, expressing low levels of ICAM-1, may trigger the recognition of the tumor and subsequent activation of the immune system, potentially causing the activated immune effectors to contribute to the regression of the tumor. Indeed, when mice were injected s.c. with anti-ICAM-1-specific antibody 1, 2, 3, 5 and 7 days after the Hepa1-6-1 administration, tumor growth was apparently suppressed (Figure 1f). However, we could not detect any Hepa1-6-1-specific cytotoxic cells within the regressed tumors (data not shown). Thus, blocking of ICAM-1 expression on Hepa1-6-1 seems to inhibit tumor growth *in vivo* probably through direct inhibition of ICAM-1 by specific antibody rather than activating antitumor T-cell response. Similar result induced by ICAM-1-specific antibody has been reported with human uveal melanoma cells.[25]

As the regression of Hepa1-6-2 may be mediated by the acquired immune system, particularly by CD8$^+$ CTLs, we examined the effect of CD8$^+$ T-cell depletion in C57BL/6 mice following two sequential intraparitoneal injections of anti-Lyt2 (3.155; rat IgM). We confirmed that the primary effector cells mediating tumor regression were CD8$^+$ T cells (Figure 1g); however, the growth of the Hepa1-6-1 tumor was not affected by CD8$^+$ T-cell depletion (data not shown). Therefore, the *in vivo* growth of Hepa1-6-2 cells can be controlled by CD8$^+$ CTLs, whereas *in vivo* immune escape by Hepa1-6-1 cells may be because of the inactivation or ignorance of CD8$^+$ CTLs in the tumor-bearing mice.

CD8$^+$ TILs within Hepa1-6-1 cells fail to become activated or demonstrate cytotoxicity

Next, we investigated the characteristics of TILs in mice injected s.c. with either the Hepa1-6-1 or Hepa1-6-2 hepatoma cell line. At 5, 7, 9 and 12 days following injection, tumor masses were excised and digested with collagenase until single-cell suspensions were obtained. The surface markers on the obtained cells were then analyzed using flow cytometry. As CD45$^+$ cells were presumably infiltrating cells and not proliferated tumor cells, gates were set to include CD45$^+$ cells within the TILs. Both tumors had been infiltrated by TILs expressing similar surface markers, except that TILs in the Hepa1-6-2-associated cells consistently had greater proportions of CD69^{high}-activated CD8β$^+$ T cells, whereas most of the CD8β$^+$ T-lymphocyte infiltrates in Hepa1-6-1 cells retained non-activated CD69^{low} phenotypes (Figure 2a). These data indicate that tumor escape was not because of the ignorance of CD8β$^+$ T cells but was rather likely because of the inactivation of these cells. Activated CD8β$^+$ TILs within Hepa1-6-2 tumor masses (TIL2) obtained 9 days after the tumor implantation were further investigated. These cells secreted both interferon (IFN)-γ and granzyme B (Figure 2b, right panel), and, when incubated overnight with IL-2, these cells demonstrated specific cytotoxicities against not only Hepa1-6-2 but also Hepa1-6-1 tumor cells (Figure 2c). Although CD8$^+$ TILs within Hepa1-6-1 tumors produced lower amounts of IFN-γ, these cells did not secrete granzyme B (Figure 2b, left panel) and showed no cytotoxicities against either tumor, even in the presence of interleukin (IL)-2 (Figure 2c). Therefore, CD8$^+$ T-cell infiltrates within the Hepa1-6-1 tumor microenvironment

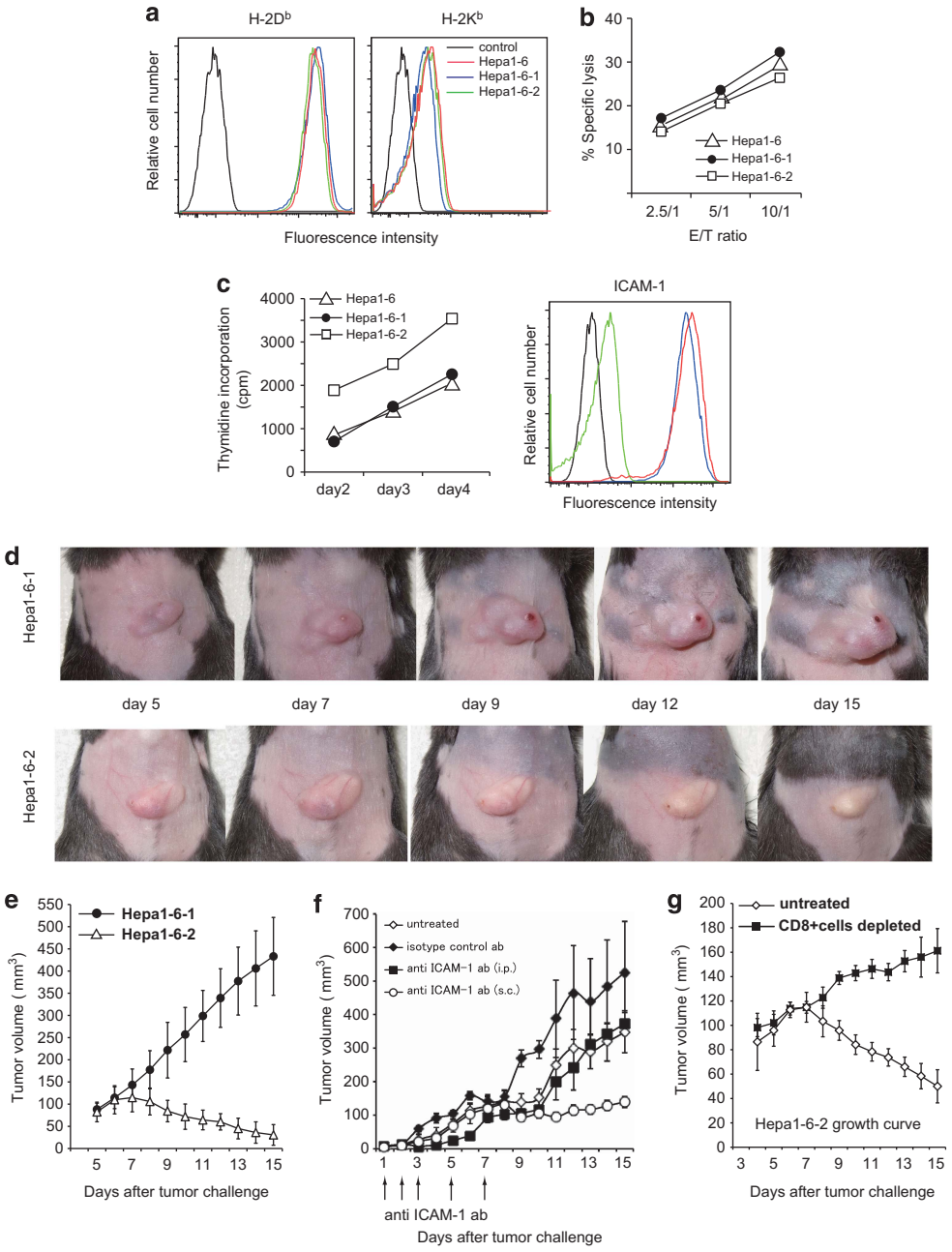


Figure 1 Analysis of two distinct hepatoma cell lines, Hepa1-6-1 and Hepa1-6-2, established from the known murine hepatoma cell line, Hepa1-6. (**a**) To confirm whether the established cell lines Hepa1-6-1 and Hepa1-6-2 expressed similar class I MHC molecules, the established lines were stained with antibodies against H-2K^b or H-2D^b. Blue lines indicate Hepa1-6-1, green lines Hepa1-6-2, red lines original Hepa1-6 and black lines isotype controls. (**b**) To determine whether the established cell lines or original Hepa1-6 could be eliminated by Hepa1-6-specific CD8+ CTLs *in vitro*, a standard ^{51}Cr-release assay against the tumor cells was performed. (**c**) To evaluate the rate of growth of the established cell lines, a standard ^{3}H-thymidine uptake assay was performed (left panel). Additionally, to examine the expression of adhesion molecules, such as ICAM-1, the established cell lines were stained with an anti-ICAM-1-specific mAb (right panel). Blue line indicates Hepa1-6-1, green line Hepa1-6-2, red line Hepa1-6 and black line isotype controls. (**d**) To observe the growth kinetics for both tumors, 1×10^7 tumor cells were s.c. implanted into syngeneic C57BL/6 mice (six per group) in the abdominal region. (**e**) To estimate the volume of the growing tumor mass, the diameter of both length (*a*) and width (*b*) were measured every day until day 15 after the implantation, and the tumor volume (*V*) was calculated according to the formula $V = ab^2/2$. Data are shown as the mean ± s.e.m. of mice per group ($n = 6$). (**f**) Mice were injected s.c. (open circle) or intraperitoneally (i.p.) (closed square) with anti-ICAM-1-specific antibody 1, 2, 3, 5 and 7 days after the Hepa1-6-1 administration, and the tumor volume (*V*) was pursued. Data are shown as the mean ± s.e.m. of mice per group ($n = 6$). (**g**) To confirm that the effector cells mediating tumor regression were primarily CD8$^+$ cells, C57BL/6 mice were i.p. injected twice with 400 μg anti-Lyt2, and then 1×10^6 Hepa1-6-2 cells were s.c. implanted and monitored for volume through day 15. Data are shown as the mean ± s.e.m. of mice per group ($n = 6$).

were weakly activated to secrete IFN-γ but failed to acquire cytotoxicity, whereas CD8$^+$ T cells within Hepa1-6-2 tumors were activated to secrete IFN-γ and granzyme B and exhibited significant cytotoxicities against both Hepa1-6-1 and Hepa1-6-2 cells. These results suggest that granzyme B production by CD8$^+$ TILs within the tumor microenvironment is a critical factor for specific cytotoxicities against tumors.

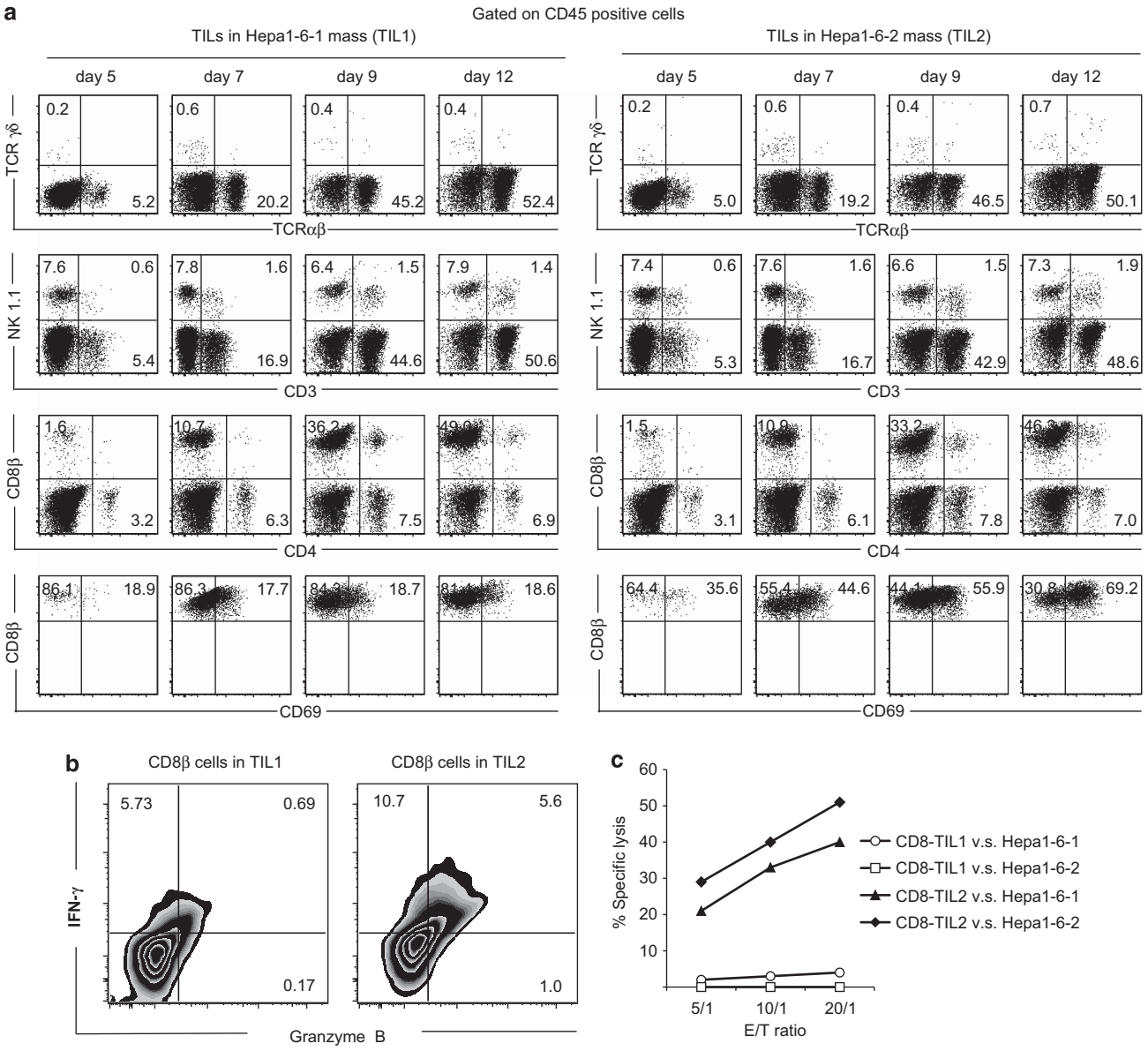


Figure 2 Percentages of CD69$^+$-activated TILs within Hepa1-6-1 cells (left 4 lines) and Hepa1-6-2 cells (right 4 lines). (**a**) At 5, 7, 9 and 12 days after implantation, emerged tumor masses were excised and digested with collagenase, and single-cell suspensions were obtained. CD45$^+$ cells were considered to be infiltrating cells but not tumor-derived cells, and thus further kinetic analyses on cell-surface markers were performed by including CD45$^+$ cells within the TIL gates; line 1, TCRαβ and TCRγδ; line 2, NK1.1 and CD3; line 3, CD8β and CD4; line 4, CD69 percentage among CD8β$^+$ cells. Representative plots from six independent experiments are shown. To evaluate the functional differences between Hepa1-6-1-associated CD8$^+$ TILs and Hepa1-6-2-associated TILS, both types of tumor cells were s.c. injected into different groups of syngeneic C57BL/6 mice. (**b**) Ten days after tumor implantation, the tumor-bearing mice were injected i.p. with 250 ng of brefeldin-A to stop intracellular trafficking. Four hours later, TILs were immediately isolated, stained for surface CD8β expression, and then fixed and permeabilized for intracellular staining with PE-conjugated anti-IFN-γ and FITC-conjugated anti-granzyme B. Representative plots from six independent experiments are shown. (**c**) Ten days following tumor implantation, emerged tumor masses were excised and digested with collagenase, and single-cell suspensions were obtained. Immediately, TIL suspensions were incubated with PE-labeled anti-CD8, followed by a PE-selecting cocktail and nanoparticles, and the samples were positively sorted using an immunomagnetic system, after which CD8$^+$ TILs from both groups were obtained. The obtained CD8$^+$ TILs were further incubated with IL-2 at 37 °C for 24 h, and a standard ^{51}Cr-release assay on the tumor cells was performed. Representative plots from six independent experiments are shown.

Downregulation of costimulatory molecule expression on TIDCs within Hepa1-6-1 tumor masses

DCs that can cross-present externally captured tumor antigens in association with MHC-I and costimulatory molecules stimulate CD8$^+$ T cells to become activated, functional CD8$^+$ CTLs. As we detected functional CD8$^+$ T cells within the Hepa1-6-2 tumor mass, we hypothesized that TIDCs can directly activate CD8$^+$ TILs within the tumor mass. We thus compared the phenotypes and functions of TIDCs within TILs for both tumors.

We found a number of CD11c$^+$ MHC-II$^+$ TILs that had infiltrated into the mass of both Hepa1-6-1 (TIL1) and Hepa1-6-2 tumors (TIL2) (Figure 3a). These TIL populations were further divided into two subpopulations, CD11c^{high} MHC-II^{high} and CD11c^{int} MHC-II^{low} cells (Figure 3b, left two panels). Using a

magnetic separation kit, we confirmed that these TILs could be classified into CD11c^{high} MHC-II^{high} DC-subset and CD11c^{+} MHC-II^{low} cells. The majority of the CD11c^{high} MHC-II^{high} population was composed of DEC-205 (CD205)$^{+}$ DCs, which can cross-present captured antigens via MHC-I to prime tumor-specific MHC-I-restricted CD8^{+} CTLs (Figure 3b, middle two panels), whereas the CD11c^{int} MHC-II^{low} cells were DEC-205$-$ (Figure 3b, right two panels).

In addition, we found that the major functional CD11c^{high} MHC-II^{high} DEC-205^{+} population within both TIDC1 and TIDC2 was from CD11b^{+} LY6C^{-} tissue-resident DCs,[26] and many CD11b^{+} LY6C^{+} inflammatory DCs were also seen in that population (Figure 3c; left panels), although such CD11b^{+} LY6C^{-} tissue-resident population was not seen within CD11c^{int} MHC-II^{low} TILs (Figure 3c; right panels). Moreover, as shown in Figure 3d, PD-L1 expression level on the CD11c^{high} MHC-II^{high} DC of TIDC1 was almost equal to that of TIDC2, indicating that the difference of specific lysis by Hepa1-6-specific CTLs between TIDC1 and TIDC2 was not attributed to the inhibitory receptors such as PD-L1.

As the percentage of CD11c^{high} MHC-II^{high} DEC-205^{+} DCs in TILs for both tumors was similar, we next compared the expression of various costimulatory molecules, including CD40, CD80 and CD86, on CD11c^{high} MHC-II^{high} DCs in Hepa1-6-1 and Hepa1-6-2 tumor masses. We found that all of the costimulatory molecules examined, particularly CD86, were downregulated in CD11c^{high} MHC-II^{high} DC subsets in Hepa1-6-1-derived TIDCs (TIDC1) to a greater extent than those on Hepa1-6-2-derived (TIDC2) cells (Figure 3e). These findings suggest that the inactivation of CD8^{+} CTLs might be caused by the impairment of the antigen-presenting capacity of TIDC1 in the Hepa1-6-1 tumor mass and that the tissue-resident DC dysfunction may allow the Hepa1-6-1 mass to grow *in vivo*.

CD11c^{high}MHC-II^{high} DEC-205^{+} tolerogenic DC subsets within Hepa1-6-1 tumors may suppress the induction of CD8^{+} CTLs from Hepa1-6-2-primed T cells

Owing to the observed DC dysfunction, we speculated that TIDC1 with downregulated costimulatory molecules may lose their ability to cross-present captured tumor antigens in association with MHC-I. To examine this possibility, the CD11c^{high} MHC-II^{high} DEC-205^{+} DC subset was enriched and labeled with ^{51}Cr for 30 min; the cells were then further cocultured with the Hepa1-6-specific CD8^{+} CTL line for an additional 4 h, after which the radioactivity present in the samples was measured to determine whether TIDC could present tumor-derived epitopes in conjunction with MHC-I. Although TIDC2s were depleted by the Hepa1-6-specific CD8^{+} CTL line at levels comparable to the positive control target Hepa1-6 (Figure 4a), less than half of TIDC1s were killed by the CTL line, indicating that the cross-presenting ability of captured tumor antigens was disrupted in TIDC1. Therefore, CD11c^{high} MHC-II^{high} DEC-205^{+} TIDC1 with downregulated costimulatory molecules may be impaired in their cross-presenting capacity.

Next, TIDCs were cocultured for 4 days with carboxyfluorescein diacetate succinimidyl ester-labeled, primed splenic T cells from mice pre-immunized with either Hepa1-6-1 or Hepa1-6-2 cells, and the cocultured cells were then harvested and analyzed using flow cytometry by gating CD8β cells. Although naive T cells from unprimed mice or Hepa1-6-1-primed mice were not activated by stimulation with Hepa1-6-1-associated TIDCs (TIDC1) (Figure 4b, left upper and middle panels) or by Hepa1-6-2-associated TIDCs (TIDC2) (Figure 4b, left lower and middle lower panels), the Hepa1-6-2-primed splenic T cells were strongly activated, demonstrating greater CD69 expression and more number of cell divisions when stimulated with TIDC2 (Figures 4b and c, right lower panel and lower panel, repectively). In contrast, the Hepa1-6-2-primed T cells were weakly activated, demonstrating less divisions when stimulated with TIDC1 (Figure 4b and c, right middle panel middle panel). Moreover, the TIDC2-activated, Hepa1-6-2-primed T cells showed equal levels of specific cytotoxicity against both Hepa1-6-1 and Hepa1-6-2 cells (Figure 4d, lower panel), although TIDC1-stimulated, Hepa1-6-2-primed T cells showed no specific cytotoxicity (Figure 4d, middle panel).

These findings suggest that CD8^{+} T cells can be primed by Hepa1-6-2 -associated TIDC2 expressing high levels of costimulatory molecules but not by Hepa1-6-1-associated TIDC1 expressing low levels of costimulatory molecules and that Hepa1-6-2-primed T cells can be activated by TIDC2 but not by TIDC1 to become cytotoxic. Although TIDC1 can present tumor antigens to Hepa1-6-2-primed T cells to initiate cell division, TIDC1 cannot induce these cells to become functional CTLs.

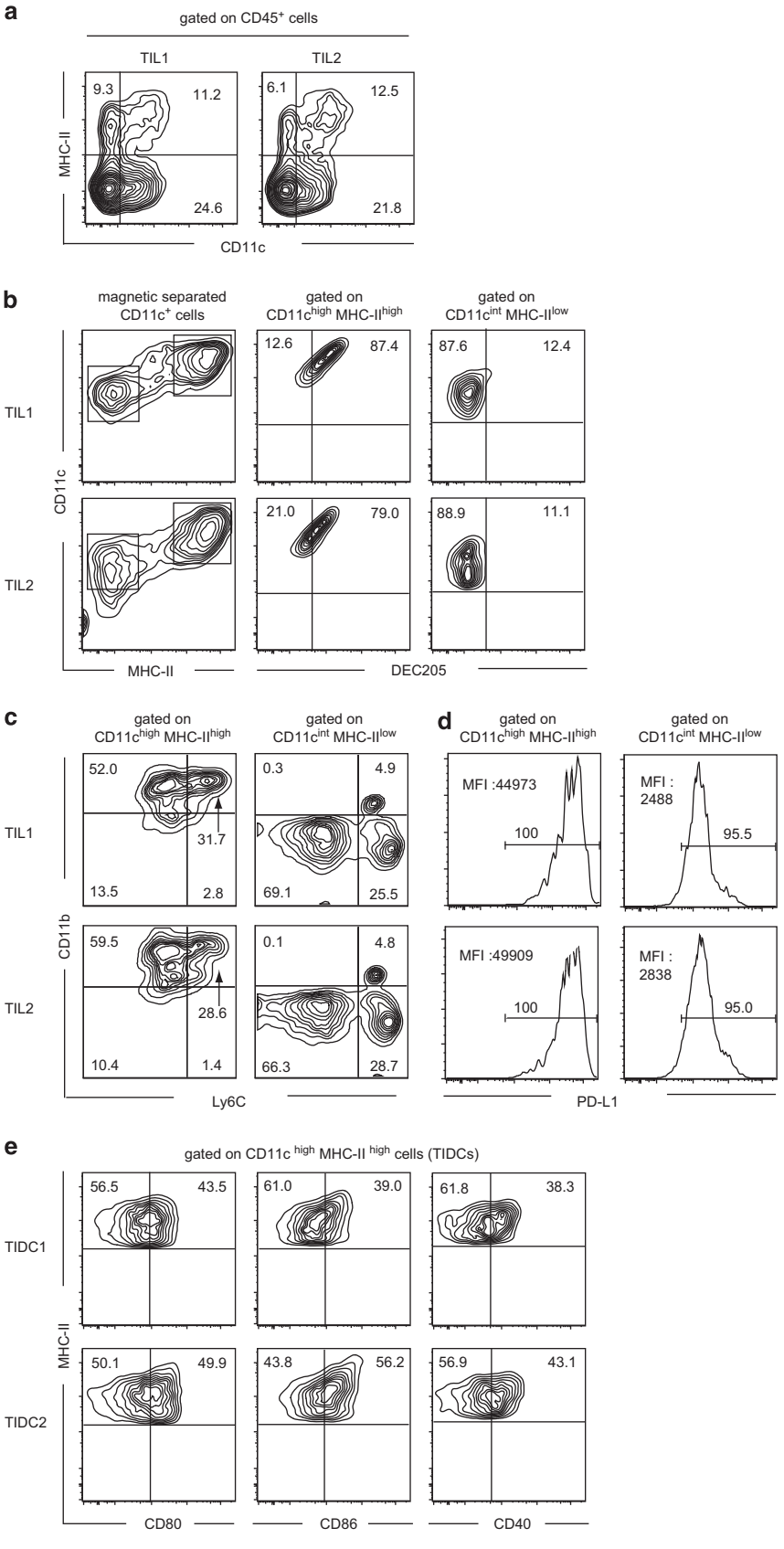
Collectively, these results suggest that CD11c^{high} MHC-II^{high} DEC-205^{+} tolerogenic DC subsets with impaired cross-presenting capacities and low costimulatory molecule expression within Hepa1-6-1 masses may suppress the induction of CD8^{+} CTLs and thus enhance tumor cell survival *in vivo*.

Hepa1-6-1-derived factors downregulate costimulatory molecule expression on DCs

Recently, several studies have shown that some cytokines and growth factors secreted by tumors, such as IL-10, IL-6, transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF), can inhibit DC maturation.[18,27] As it has been reported that hepatoma cells can secrete α-fetoprotein (AFP), VEGF and TGF-β,[28] we thus measured the levels of these factors in culture supernatants. Indeed, significantly greater amounts of AFP, VEGF and TGF-β were detected in the supernatant of Hepa1-6-1 cells, whereas only small amounts of these molecules were detected in the Hepa1-6-2-derived supernatants (Figure 5a). We then examined whether the culture supernatant from tumors could inhibit the maturation of bone marrow-derived immature DCs *in vitro*. Bone marrow-derived immature DCs from syngeneic C57BL/6 mice were cocultured with conditioned medium containing 10% culture supernatants of either Hepa1-6-1 or Hepa1-6-2 cells for 5 days with granulocyte–monocyte colony-stimulating factor (GM-CSF). The culture supernatant from Hepa1-6-1 significantly suppressed costimulatory molecular expression of CD86 and, to a lesser extent, CD80 and CD40 on bone marrow-derived immature DC, although the expression of these molecules was not affected by the supernatant from Hepa1-6-2 cells (Figure 5b). These results indicate that such tumor-derived factors may cause neighboring DCs to downregulate the expression of costimulatory molecules, causing the priming of tumor-specific MHC-I-restricted CD8^{+} CTLs to be impaired.

Effect of Hepa1-6-2 implantation on Hepa1-6-1 growth *in vivo*

Finally, we examined the effect of simultaneous co-implantation of Hepa1-6-1 cells in the right abdominal region and Hepa1-6-2 cells in the left abdominal region on the growth of both tumors. Hepa1-6-1 cells established a tumor mass, whereas Hepa1-6-2 cells ceased growing around day 7 (Figure 6a). We observed activated CD69^{high} CD8^{+} CTLs (left 2 panels) with the capacity to eliminate both Hepa1-6-1 and Hepa1-6-2 cells (right panel) within the Hepa1-6-2-associated compartment (TIL2) but not within the Hepa1-6-1 mass (TIL1) on day 9 (Figure 6b). These results indicate that CD8^{+} CTLs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gated on CD45+ cells
TIL1
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MHC-II
CD80
CD86
CD40

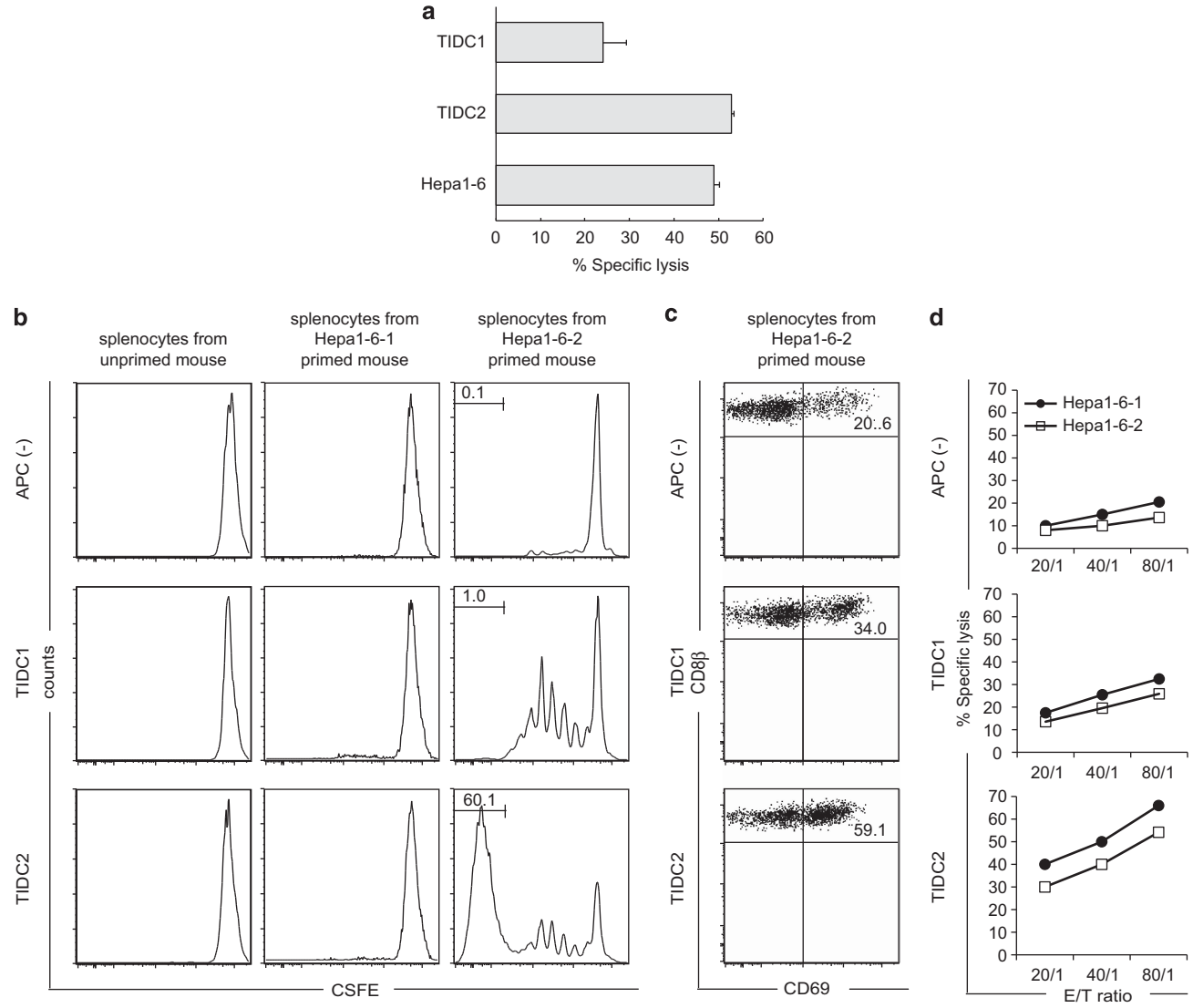

Figure 4 Effect of CD11c^{high} MHC-II^{high} DEC-205^{+} DC subsets within either Hepa1-6-1 or Hepa1-6-2 tumor masses on the induction of CD8^{+} CTLs from primed splenic T cells. (**a**) Hepa1-6-specific CD8^{+} CTLs were incubated with 3×10^{3} ^{51}Cr-labeled CD11c^{high} MHC-II^{high} DEC-205^{+} TIDC1, TIDC2 and Hepa1-6 cells for 4 h at 37 °C in round-bottom 96-well cell culture plates. After incubation, the plates were centrifuged for 10 min at $330 \times g$, and 100 µl of cell-free supernatants was collected to measure radioactivity. Data are shown as the mean ± s.e.m. of cells per group ($n = 4$). (**b**) Carboxyfluorescein diacetate succinimidyl ester-labeled splenic T cells from unprimed mice (left three panels), Hepa1-6-1-primed mice (middle vertical three panels) or Hepa1-6-2-primed mice (right three panels) were stimulated for 4 days with CCM alone (upper three panels), with Hepa1-6-1-associated CD11c^{high} MHC-II^{high} DC (TIDC1) (middle horizontal three panels) or with Hepa1-6-2-associated CD11c^{high} MHC-II^{high} DC (TIDC2) (lower three panels). The cocultured cells were then harvested, gated to CD8β cells and analyzed to determine the number of cell divisions using flow cytometry. (**c**) Hepa1-6-2-primed splenic T cells were stimulated for 4 days with CCM alone (upper panel), with TIDC1 (middle panel), or with TIDC2 (lower panel), and CD69 expression among CD8^{+} cells was analyzed. (**d**) TIDC2-activated Hepa1-6-primed T cells showed specific cytotoxicity against both Hepa1-6-1 and Hepa1-6-2 cells at equal rates (lower panel), whereas TIDC1-activated Hepa1-6-primed T cells showed no cytotoxicity against either Hepa1-6-1 or Hepa1-6-2 cells (middle panel), similar to the control (upper panel). Representative graphs from five independent experiments are shown.

Figure 3 Costimulatory molecule expression on TIDCs within Hepa1-6-1 tumor masses is downregulated. (**a**) A number of CD11c^{high} MHC-II^{high} DCs among the CD45^{+} population infiltrated into the masses of both Hepa1-6-1 (TIL1) and Hepa1-6-2 (TIL2) tumors. (**b**) These populations were further divided into two subpopulations: CD11c^{high} MHC-II^{high} and CD11c^{int} MHC-II^{low} (as indicated in the squares of the left two panels). The majority of the CD11c^{high} MHC-II^{high} population was composed of DEC-205^{+} DCs (middle two panels), whereas the CD11c^{int} MHC-II^{low} cells were largely DEC− (right two panels). The percentage of CD11c^{high} MHC-II^{high} DEC-205^{+} DCs in TILs for both tumors was approximately equal. (**c**) CD11b and Ly6C expression on CD11c^{high} MHC-II^{high} TIDCs to see whether they originate from tissue-resident or from inflammatory DCs. (**d**) The expression of PD-L1 on CD11c^{high} MHC-II^{high} DCs in Hepa1-6-1 (TIDC1) and Hepa1-6-2 (TIDC2) tumor masses was examined. Representative graphs from six independent experiments are shown. (**e**) The expression of the costimulatory molecules CD40, CD80 and CD86 on CD11c^{high} MHC-II^{high} DCs in Hepa1-6-1 (TIDC1) and Hepa1-6-2 (TIDC2) tumor masses was examined. Representative graphs from six independent experiments are shown.

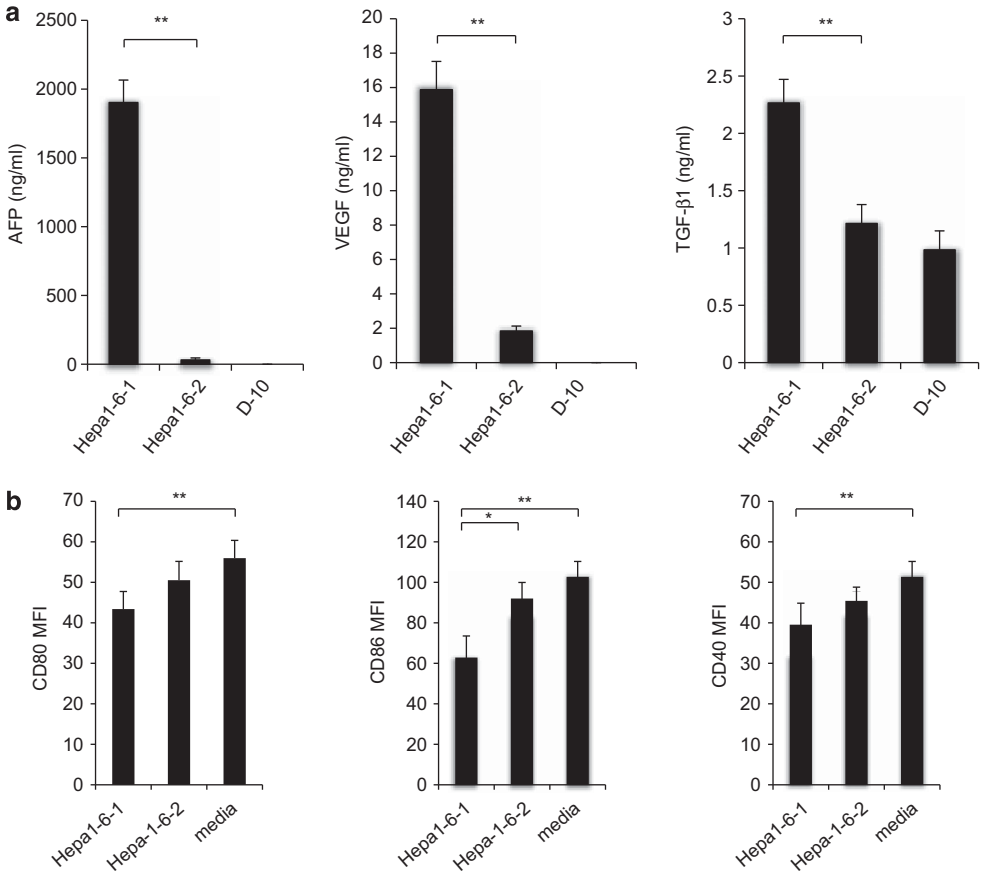

Figure 5 Hepa1-6-1-derived factors downregulate costimulatory molecule expression on DCs. (**a**) The amounts of AFP, VEGF and TGF-β in the culture supernatant of Hepa1-6-1 or Hepa1-6-2 cells were measured. Data are shown as the mean ± s.e.m. per group ($n=5$). (**b**) One million bone marrow-derived immature DCs (BM-iDCs) from syngeneic C57BL/6 mice were cocultured with 10% conditioned medium from Hepa1-6-1 or Hepa1-6-2 cells for 5 days with GM-CSF and subsequently evaluated for the expression of CD80, CD86 and CD40. Data are shown as the mean ± s.e.m. of cells per group ($n=5$). $^{*}P<0.05$, $^{**}P<0.005$, Student's *t*-test.

induced within Hepa1-6-2 tumor masses do not affect neighboring Hepa1-6-1 growth. Next, we injected Hepa1-6-2 cells into the right abdominal region and subsequently implanted Hepa1-6-1 cells in the same (right) side or in the opposite (left) side at various time points. More than 13 days after Hepa1-6-2 implantation, inoculated Hepa1-6-1 cells did not grow in either site (Figure 6c). These findings suggest that Hepa1-6-1 growth can be regulated by Hepa1-6-2-specific $CD8^+$ CTLs with crossreactive cytotoxicity against Hepa1-6-1 cells only when these CTLs are established before tumor cell implantation; otherwise, inoculated or intruder $CD8^+$ CTLs may be inactivated by tolerogenic DCs within the tumors.

DISCUSSION

In this study, we established two distinct hepatoma cell lines, Hepa1-6-1 and Hepa1-6-2, from Hepa1-6 cells. After s.c. implantation, Hepa1-6-1 cells formed a solid tumor mass, whereas Hepa1-6-2 cells regressed after 1 week of temporal growth. Tumor regression was caused by specific cytotoxicity mediated through $CD8^+$ CTLs in the TILs (TIL2) that may have been stimulated by $CD11c^{high}$ MHC-II^{high} DEC-205^+ TIDC2 with cross-presenting capacity. The finding that $CD8^+$ CTLs induced within the TILs killed both Hepa1-6-1 and Hepa1-6-2 cells to a similar extent *in vitro* indicates that both tumor cells expressed the same antigenic epitope(s) in association with MHC-I molecules. Moreover, tolerogenic $CD11c^{high}$ MHC-II^{high} DEC-205^+ TIDCs (TIDC1) with low levels of costimulatory molecules and impaired cross-presenting abilities were observed within the Hepa1-6-1 tumor mass.

Thus, TIDC1 failed to prime specific $CD8^+$ CTLs *in vivo*, although these cells could weakly initiate cellular division of primed $CD8^+$ T cells, as shown in Figure 4b (middle panel). Although we have performed experiments in which the memory splenocytes obtained from Hep1-6- or Hepa1-6-2-immunized mice were cocultured either with TIDC1 and TIDC2 or with TIDC1 and Hepa1-6-2 to see whether TIDC1 suppress the activation of immune splenocytes, we could not confirm the suppressive effect of TIDC1. Thus, Hepa1-6-1 might directly tolerise or fail to amplify TILs. Such a priming failure by $CD11c^{high}$ MHC-II^{high} DEC-205^+ TIDC1 within the Hepa1-6-1 tumor mass may be caused by the downregulation of costimulatory molecules and impaired cross-presentation ability induced by Hepa1-6-1-dependent secreted factors such as AFP, VEGF and TGF-β. Indeed, it has recently been reported that tumor-produced IL-6, TGF-β1, IL-10 and VEGF can cause APCs to display a tolerogenic phenotype, characterized by the lower expression of costimulatory molecules.[18,27] These tumor-induced tolerogenic APCs can be stimulated by IFN-γ secreted via Th1 cells or by other stimuli[24] within the tumor environment.

The Hepa1-6-1 cell line with high ICAM-1 expression was established using D-10 media, whereas Hepa1-6-2 cells with

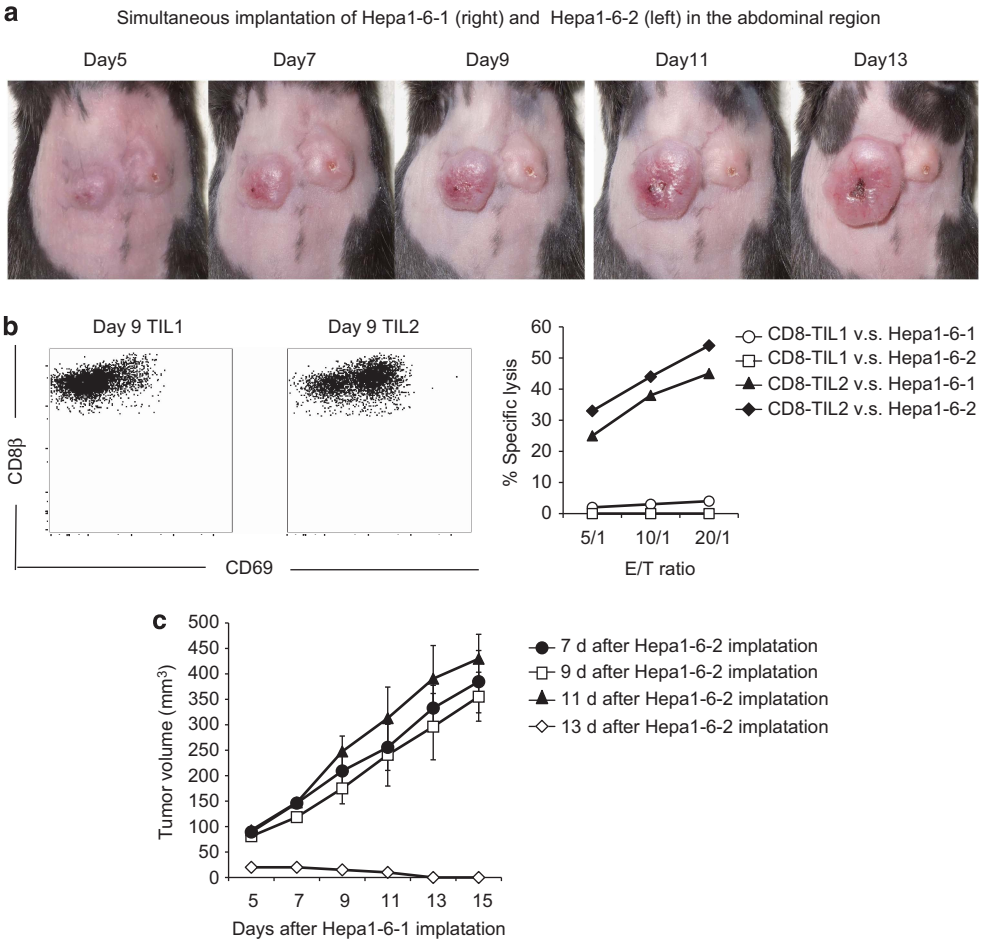

Figure 6 Effect of simultaneous implantation or pre-administration of Hepa1-6-2 cells on Hepa1-6-1 tumor growth. (**a**) The effect of simultaneous co-implantation of Hepa1-6-1 in the right abdominal region and Hepa1-6-2 in the opposite left abdominal region on tumor growth was examined. Representative data from four independent experiments are shown. (**b**) CD69 expression and cytotoxic activity of $CD8^+$ TILs within Hepa1-6-1 and Hepa1-6-2 masses were examined 9 days after co-implantation. Representative data from four independent experiments are shown. (**c**) Effect of pre-administration of Hepa1-6-2 cells in the right abdominal region on the growth of Hepa1-6-1 implantation in the same (right) side or in the opposite (left) side was analyzed at various time points. Data are shown as the mean ± s.e.m. of mice per group ($n=4$).

downregulated ICAM-1 expression were obtained using R-10. D-10 has a higher glucose concentration (4500 mg l^{-1}), whereas R-10 has a lower concentration (2000 mg l^{-1}). The capacity of tumor cells to form a tumor mass *in vivo* may correlate with their capacity to attach to neighboring cells via adherent molecules, such as ICAM-1. Therefore, Hepa1-6-2 may have lost the ability to form a tumor mass, as well as the ability to downregulate costimulatory molecules on the associated TIDCs and induce their tolerogenicity; thus, tumor-specific $CD8^+$ CTLs were elicited among TILs. In contrast, as demonstrated in Figure 1f, similar to a recent finding,[25] the growth of Hepa1-6-1 expressing higher ICAM-1 could be inhibited directly by injecting ICAM-1-specific antibody *in vivo* without inducing specific immune effectors. These findings suggest that we can elicit tumor-specific cell-mediated immunity through the selective activation of costimulatory molecules on the tolerogenic DCs, rather than through the downregulation of ICAM-1 on tumor masses by intravenous administration of anti-ICAM-1 antibodies or by feeding cells with low-glucose media.

As demonstrated in the present study, we detected functional $CD8^+$ CTLs within the tumor mass of Hepa1-6-2 cells, and thus we speculated that TIDCs are the key cells that directly activate $CD8^+$ TILs within the tumor. Moreover, as shown in Figure 6a, when Hepa1-6-1 was implanted in the right abdominal region and Hepa1-6-2 in the opposite left abdominal region simultaneously, Hepa1-6-1 established a tumor mass, whereas Hepa1-6-2 ceased growing around day 7. Therefore, we could detect functional $CD8^+$ CTLs with the capacity to eliminate not only Hepa1-6-2 cells but also Hepa1-6-1 among TILs in the Hepa1-6-2 tumor. Although functional $CD8^+$ CTLs should have also been observed in the Hepa1-6-1 tumor zone, regression of the Hepa1-6-1 tumor mass was not observed, suggesting that functional $CD8^+$ CTLs are inactivated within the Hepa1-6-1 mass region, possibly by tolerogenic TIDCs.

Therefore, breaking the tolerogenic state of TIDCs within the tumor-associated compartment seems to offer a useful strategy for controlling tumor growth. Indeed, it has recently been shown that depletion of DCs from the tumor microenvironment in mice bearing ovarian cancer cells markedly delayed tumor growth and enhanced the specific antitumor immune response.[22] Moreover, as we demonstrated recently, the selective activation of a specific subset of DCs, such as DEC-205^+ DCs, by depleting $33D1^+$ DCs seems to activate both $CD8^+$ CTLs and NK effectors without additional tumor-antigen stimulation *in vivo*.[3,29] It was also found that

suppression of previously established tumor growth by activated mucosal CD8$^+$ CTLs could be elicited by oral administration of tumor antigen with CT.[13] The upregulated expression of costimulatory molecules on TIDCs within regressed tumors mediated via oral administration of tumor antigen with CT has also been confirmed (Wakabayashi and Takahashi, manuscript in preparation). These findings suggest that tolerogenic DCs within tumors can be converted to functional DCs through the activation of DEC-205$^+$ DCs. Therefore, we would like to propose a new method of immunotherapy that utilizes the selective suppression, depletion[22] or even alteration[23] of tolerogenic TIDCs with downregulated costimulatory molecules within tumors. Such an immunotherapy could be achieved by activating mucosal DEC-205$^+$ DCs using a selection of adjuvants or TLR ligands[24] or by suppressing 33D1$^+$ DCs. We hope that this method will result in a new era in cancer immunotherapy.

METHODS

Mice

Six- to eight-week-old female C57BL/6 (H-2^b) mice were purchased from Charles-River, Japan, maintained in microisolator cages under pathogen-free conditions and fed autoclaved laboratory food and water. All animal experiments were performed according to the guidelines set forth for the care and use of laboratory animals by the National Institutes of Health (Bethesda, MD, USA) and approved by the Review Board of Nippon Medical School (Tokyo, Japan).

Culture medium

The medium used for culturing immunocompetent cells, such as CTLs and DCs, was RPMI1640 (Sigma-Aldrich, St Louis, MO, USA)-based complete culture medium (CCM)[30] supplemented with 50 mM 2-mercaptoethanol (2-ME) (Invitrogen Life Technologies, Carlsbad, CA, USA), 2 mM L-glutamine (Sigma-Aldrich), 1 mM sodium pyruvate (Invitrogen Life Technologies), 0.1 mM nonessential amino acids (Invitrogen), a mixture of vitamins (Invitrogen), 1 mM HEPES (Invitrogen), 100 U ml^{-1} penicillin (Invitrogen), 100 μg ml^{-1} streptomycin (Invitrogen) and 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT, USA). Tumor cells were cultured in either DMEM (Invitrogen) supplemented with 1 mM HEPES, 100 U ml^{-1} penicillin (Invitrogen), 100 μg ml^{-1} streptomycin and 10% heat-inactivated FCS (D-10) or RPMI1640 supplemented with 1 mM HEPES, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% heat-inactivated FCS (R-10).

Establishment of Hepa1-6-1 and Hepa1-6-2 tumor lines

A murine hepatoma cell line, Hepa1-6 (H-2b),[20] was purchased from the American Type Culture Collection (Rockville, MD, USA). Two murine hepatoma cell lines with distinct characteristics, Hepa1-6-1 and Hepa1-6-2, were established from Hepa1-6. Briefly, the uncontrollable tumor cell line Hepa1-6-1 was obtained from purified cells of a surgically removed tumor mass that had been grown for 3 months after s.c. implantation of Hepa1-6 cells into syngeneic C57BL/6 mice and maintained in the D-10 medium. A controllable tumor cell line demonstrating spontaneous regression, Hepa1-6-2, was obtained by the repetitive *in vitro* transfer of Hepa1-6 cells for several months in R-10.

Tumor injection and measurement of tumor size

Ten million tumor cells were injected s.c. into each mouse in the right abdominal region in 100 μl phosphate-buffered saline with a 27-gauge needle syringe. To estimate the volume of the growing tumor mass, diameters of both the length (a) and the width (b) of the mass were measured every other day through day 15, after which the tumor volume (V) was calculated according to the formula $V = ab^2/2$, as described previously.[13] When the longer axis of each tumor was >20 mm in diameter, all of the mice were anesthetized and killed according to the National Institutes of Health guidelines.

In vivo depletion of CD8$^+$ cells

For the *in vivo* depletion of CD8$^+$ cells, C57BL/6 mice were intraperitoneally injected twice (on days 1 and 3) with 400 μg Anti-Lyt2 (3.155; rat IgM).[31] Flow cytometry analysis confirmed that $>95\%$ of CD8$^+$ T cells in the spleen had been depleted.

Isolation of TILs

For the isolation of TILs, tumors were excised from mice and digested with 1 mg ml^{-1} collagenase (Wako Pure Chemical Industries, Osaka, Japan) for 45 min at 37 °C, crushed gently to homogenize the mass and then filtered through nylon mesh. Obtained leukocytes were further separated from contaminating tumor cells by centrifugation over a 40–75% Percoll gradient at $600 \times g$ for 18 min at room temperature to obtain purified TILs.

Purification of CD11c$^+$ TILs

To purify tumor-infiltrating CD11c$^+$ cells, TIL suspensions were incubated with PE-labeled anti-CD11c, followed by a PE-selecting cocktail and nanoparticles; cells were then positively selected using an immunomagnetic system (StemCell Technologies, Vancouver, Canada), which yielded a population containing $\sim 95\%$ CD11c$^+$ TILs.

Restimulation of Hepa1-6-primed lymphocytes with CD11c^{high} TIDCs

One million Hepa1-6-1 or Hepa1-6-2 cells in 200 μl of phosphate-buffered saline were s.c. injected into syngeneic C57BL/6 mice with a 27-gauge needle syringe. Two weeks after the inoculation, primed splenic T cells were obtained using a nylon wool column labeled with 5 mM carboxyfluorescein diacetate succinimidyl ester,[32] and further cocultured with 1×105 CD11c^{high} TIDCs in 200 μl of CCM in a 96-well flat plate for 2–4 days. Next, the restimulated cells were harvested and stained with various antibodies including CD8β, and CD8β-gated cells were further analyzed using fluorescence-activated cell sorting (FACS) to determine the number of cell divisions, which correlates with the ability of the TIDCs to prime T cells.

Effect of hepatoma cell culture supernatants on bone marrow (BM)-derived DCs

First, BM cells prepared from femurs and tibias of syngeneic C57BL/6 mice were depleted of red blood cell using ammonium chloride, as described previously.[33] Next, one million BM cells were plated in 24-well culture plates and incubated in CCM supplemented with 3.3 ng ml^{-1} of murine rGM-CSF (Genzyme Techne, Minneapolis, MN, USA). On day 2 of culture, floating cells were gently removed, and fresh CCM was added with various concentrations of conditioned medium obtained from tumors. On day 5, nonadherent cells were collected and analyzed using FACS.

In vivo analysis of tumor-specific T-cell production of IFN-γ and granzyme B

Tumor-bearing mice were injected intraperitoneally with 250 ng of brefeldin-A (Sigma-Aldrich). Four hours later, TILs were immediately isolated, stained for surface molecule expression, and then fixed and permeabilized for intracellular staining with PE-conjugated anti-IFN-γ and FITC-conjugated anti-granzyme B (eBioscience, San Diego, CA, USA).

CTL assay

A standard ^{51}Cr-release assay was performed to determine the cytotoxicity of the purified CD8$^+$ TILs or of the Hepa1-6-specific immune cells as previously described.[3] In brief, various numbers of effector cells were incubated with 3×10^3 ^{51}Cr-labeled target cells for 4 h at 37 °C in 200 μl RPMI1640 medium containing 10% heat-inactivated FCS in round-bottom 96-well cell culture plates (BD Biosciences, San Diego, CA, USA). After incubation, the plates were centrifuged for 10 min at $330 \times g$, and 100 ml of the cell-free supernatants was collected to measure the radioactivity with a Packard Auto-Gamma 5650 counter (Hewlett-Packard, Tokyo, Japan). Maximum release was determined from the supernatant of cells that had been lysed by the addition of 5% Triton X-100, and spontaneous release was determined from target cells incubated

without added effector cells. The percentage of specific lysis was calculated as $100 \times$ (experimental release − spontaneous release)/(maximum release − spontaneous release).

Cytokine detection

Established hepatoma cells ($1 \times 10^5\,ml^{-1}$) were cultured in D-10 medium for 3 days. The amounts of mouse VEGF and TGF-β1 in the culture supernatants were determined using ELISA kits (R&D Systems, Minneapolis, MN, USA), and AFP was measured using a standard ELISA assay with alkaline phosphatase-conjugated anti-mouse AFP-specific antibody.

Antibodies and flow cytometry analysis

The following antibodies were purchased from Biolegend (San Diego, CA, USA): anti-CD3-FITC (145-2C11), anti-CD11c-FITC (N418), anti-NK1.1-PE (PK136), anti-PD-L1-PE (10F.9G2), anti-ICAM-1-APC (YN1/1.7.4), anti-CD8α-APC (53–6.7), anti-I-A/I-E-APC (M5/114.15.2), anti-H-2Kb-biotin (AF6-88.5), anti-H-2Db-biotin (KH95), anti-Ly6C-biotin (HK1.4) and anti-CD45-biotin (30-F11). Anti-TCRβ-FITC (H57-597), anti-γδTCR-PE (GL3), anti-CD4-PE (RM4-5), anti-CD80-PE (16-10A1), anti-CD86-PE (GL1), anti-CD40-PE (1C10) and anti-CD8β-APC (H35-17.2) were purchased from eBioscience. Anti-CD8-FITC (CT-CD8) and anti-DEC-205-FITC (NLDC-145) were purchased from Caltag Laboratories (Burlingame, CA, USA) and Cedarlane (Toronto, Canada), respectively. Cell suspensions were stained with relevant antibodies on ice for 30 min in phosphate-buffered saline with 2% heat-inactivated FCS and 0.1% sodium azide, washed twice and analyzed with a FACSCantoII six color cytometer (BD Bioscience) using FlowJo Software (Tree Star, Inc, Ashland, OR, USA).

Statistical analysis

The results were analyzed using Student's *t*-test, and the results are presented as the mean ± s.e.m. Differences of $P < 0.05$ were considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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